Original Article

An intronic polymorphism of *NFATC1* gene shows a risk association with biopsy-proven acute rejection in renal transplant recipients

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Background: We aimed to explore the influence of single nucleotide polymorphisms (SNPs) in *NFATC1* gene on the occurrence of biopsy-proven acute rejection (BPAR) in renal transplant recipients.

Methods: Blood samples from 131 subjects with stable allograft function (STA) and 69 with BPAR episodes were collected and analyzed using target sequencing (TS) with an established panel. Odds ratios (OR) and 95% confidence intervals (95% CIs) were calculated for logistic regression models adjusted for confounding factors. Pathological changes were extracted and the relationship with tagger SNPs was calculated. Moreover, the CCK-8 assay was performed to explore the proliferation of T lymphocytes, and PCR, Western blotting and enzyme-linked immunosorbent assay were applied to identify the effect of mutant on the activation of T cells.

Results: High-quality readouts were obtained for 55 *NFATC1* SNPs and 14 tagger SNPs were remained for further analysis. After adjusting for clinical confounding factors, the distribution of four *NFATC1* SNPs, including rs2290154, rs2304738, rs754093 and rs754096, were statistically significant between STA and BPAR groups. Pathological association analysis indicated one SNP, rs2290154, was significantly related with the Banff score and renal tubulitis. Our *in vitro* study suggested that *NFATC1* rs2290154 mutant could remarkably promote the T cell proliferation, increase the transcription of *NFATC1* mRNA and expression of NFATC1 protein, as well as the interleukin-2 (IL-2) secretion.

Conclusions: We reported the crucial association of *NFATC1* gene with the occurrence of acute rejection (AR) episodes. Moreover, *in vitro NFATC1* rs2290154 was significantly involved in the T lymphocytes activation and proliferation through increasing the translation of *NFATC1* mRNA and expression of NFATC1 protein, along with the secretion of IL-2.

Keywords: *NFATC1*; biopsy-proven acute rejection (BPAR); single nucleotide polymorphisms (SNPs); kidney transplantation

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Introduction

Kidney transplantation has been considered as the optimal therapy for patients with end-stage renal diseases (1). Benefiting from novel immunosuppressive agents, incidence of acute rejection (AR) following kidney transplantation has dramatically declined over the past decades; however, approximately 10–15% of recipients currently suffer from AR during their first year post-transplant, which still adversely contribute to the loss of graft function (2-4). Recently, immunological factors, especially T cell-mediated immune responses, are believed to be strongly associated with the pathogenesis of AR episodes (5,6).

Nuclear factor of activated T cells (NFAT) was originally observed in the nuclear extracts from activated T cells and was reported to activate T cells by binding to the interleukin-2 (IL-2) promoter (7). Moreover, with regard to the extensive administration of cyclosporine A (CsA) and tacrolimus (TAC), which function by inhibiting calcineurin activity and NFAT nuclear translocation, as well as the consequent target gene transcription, the role of NFAT has been considered essential in the context of solid organ transplantation (8). The NFAT family of transcription factor consists of five members (*NFATC1–NFATC5*), which are all coded by different genes (9). In addition to *NFATC5*, which is activated in response to osmotic stress, all other NFAT proteins are regulated by the calcium and calcineurin signaling pathways (10-12). The calcium-related NFAT proteins consist of two conserved domains: (I) the Rel-homology region (RHR); and (II) the NFAT homology region (NHR), which is a regulatory domain containing a calcineurin-binding site that regulates calcium, as well as calcium and calcineurin signaling pathways (13,14). Moreover, the NHR is highly phosphorylated under steady-state condition, maintaining NFAT in an inactive state and restricted to the cytoplasm; NHR can be dephosphorylated under conditions of increased intracellular calcium levels and the activation of calcineurin, leading to NFAT cytoplasmic-nuclear trafficking (15,16). Once in the nucleus, NFAT proteins function as transcription factors by binding to their target promoter regions (12).

The NFATC1 proteins are encoded by *NFATC1* gene and located on chromosome 18q23. In addition, mice lacking the NFATC1 protein develop splenomegaly associated with the hyperproliferation of both B and T cells, combined with altered levels of IL-4, IL-13, and granulocyte-monocyte colony stimulating factor (GM-CSF), indicating an essential role of *NFATC1* on immune cell growth and transcription regulation (17). In solid organ transplantation, the regulation of gene expression (e.g., IL-2 and GM-CSF) by *NFATC1* has been correlated with an over-immunosuppressive status, and is also observed in recipients who receive calcineurin inhibitors (CNIs) (18-20). While these studies illustrate the extensive relationship between NFATC1 proteins and the immune response, no studies have explored its influence on the AR episodes that occur following solid organ transplantation.

In this study, we performed a comprehensive analysis of *NFATC1* single nuclear polymorphisms (SNPs) in renal transplant recipients using target sequencing (TS) based on next-generation sequencing (NGS), and 55 SNPs were detected. Then, 14 tagger SNPs were remained for further association analysis after the Hardy-Weinberg equilibrium (HWE) and major allele frequencies (MAF) analysis. The multivariable logistic regression analysis adjusted by two confounding clinical characteristics identified one significant SNP (rs2290154) with the remarkable pathological changes. Finally, our *in vitro* study transfected the mutated plasmid of rs2290154 and demonstrated that the mutation of rs2290154 promoted T cell proliferation by causing the increasingly translation of *NFATC1* mRNA and synthesis of NFATC1 protein, which leads to the increased secretion of IL-2.

Methods

Ethics approval and consent to participate

Local ethics committee of the First Affiliated Hospital with Nanjing Medical University approved the protocols followed in this study (2016-SR-029). Written informed consents were obtained from all transplant recipients. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Recipients in this study was strictly limited to living-related transplantation of donors to lineal or collateral relatives not beyond the third degree of kinship or transplantation of kidney donors after cardiac death from 2011 to 2015.

Patients and immunosuppressive therapy

A total of 200 cases were selected among recipients who underwent kidney transplantation surgery between

1st February 2011 and 1st December 2015 at the renal transplant center of First Affiliated Hospital with Nanjing Medical University. Detailed medical records and materials, including age, gender, transplant date, duration of transplant, transplant times, immunosuppressive protocol, panel reactive antibody (PRA) and human leukocyte antigen (HLA) mismatch, were carefully extracted by two independent clinicians (Zijie Wang and Ruoyun Tan) for patient selection.

Detailed immunosuppressive protocol following renal transplant was presented in our previous study (21). Briefly, all recipients received a three- or four-drug immunosuppressive regimen consisting of CsA or TAC in combination with mycophenolate mofetil (MMF) and prednisone (Pred), with or without sirolimus. The dosage of CsA and TAC was started at 8 and 0.2 mg/kg/d, respectively, and adjusted according to the results of therapeutic drug monitoring of the serum creatinine levels. A dosage of 200 mg/d of intravenous methylprednisolone was adopted for each BPAR episode lasting 3 to 5 days. The BPAR diagnostic criteria were based on the comprehensive evaluation of histological and clinical characteristics. The clinical characteristics were comprised of a 20% increase in serum creatinine levels from baseline (not attributable to other cases), fever, urine protein, and pain in the region of the transplanted kidney; histologically, the BPAR diagnosis was confirmed by an allograft biopsy according to Banff 07 classification (22), and consequently, the i, g, t, v, and ptc scores for each case were collected, which represent the interstitial inflammation, glomerulitis, tubulitis, intimal arteritis, and peritubular capillaritis, respectively.

During the follow-up of 3 years post-transplant for each subject, recipients who experienced at least one BPAR episode were assigned to BPAR group, while recipients with stable allograft function (STA) during the follow-up were enrolled to stable control group.

Sample collection, preparation, and sequencing

The detailed procedures of sample collection and the TS steps were described in our previous study (23). In brief, peripheral blood samples (2 mL) were collected from each recipient included in our study and the DNA was extracted. Quantitative detection of the concentration and purity of genomic DNA (gDNA) was performed while the gene integrity was tested by agarose gel electrophoresis. Next, a pool containing upstream and downstream oligonucleotides specific to the targeted regions of interest was hybrids to the gDNA samples. Quantitative detection was performed and fragmentation was followed by end repair, dA tailing, and sequencing adaptor ligation with an ABI 9700 PCR instrument (ABI, USA). The adapter-ligated DNA was amplified by selective, limited-cycle PCR and then quantitatively analyzed. The prepared library (750 ng) was hybridized overnight and hybridized products were mixed with 200 μL MyOne Streptavidin T1 magnetic beads (Invitrogen, USA) for 30 min at room temperature. Mixture was amplified for 16 PCR cycles and quantitatively assessed. The captured libraries were denatured and loaded onto an Illumina cBot instrument at 12 to 16 pmol/L for cluster generation according to the manufacturer's instructions. Up to 20 WUCaMP libraries were sequenced per HiSeq lane.

Data analysis

Sequencing data, including the number of altered chromosomes, genomic alternation information, and the depth of sequencing coverage were analyzed. All analyses were based on the human reference sequence UCSC build hg19 (NCBI build 37.2) using the Burrows-Wheeler Aligner (BWA) (24). Local alignment and duplication removal were completed by the application of the Genome Analysis Tool Kit (GATK) and Picard software. SNP detection was performed using dbSNP 132. Damaging or deleterious SNPs were predicted using Gemini software. Prediction tools, including sorting intolerant from tolerant (SIFT) and polymorphism phenotyping (PolyPhen), were used to analyze all human non-synonymous SNPs. In addition, putative somatic variant calls were detected with two separate programs (MuTect 1.1.5 and VarScan 2.3.6 software) by pairing each sample with its matched blood.

Cell culture

The Jurkat T cell was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco, Australia) and 1% penicillin-streptomycin in an incubator with humidified 5% CO_2 at 37 ℃. The plasmids pCDNA3.1, pCMV-NFATC1 and pCMV-NFATC1-S617 were synthesized by Genechem (Shanghai, China) and then the transfection was performed according to the manufacturer's protocol. Detailed information of plasmids construction was presented in the *Table S1*. The cells were stimulated by PMA/Ionomycin mixture (Multi Sciences, Hangzhou, China) for 24 h among Stimulated group, pCDNA3.1 group, pCMV-NFATC1

group and pCMV-NFATC1-S617 group. The naive Jurkat T cell was defined as Control group.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cultured cells by using RNA extraction kits (Tiangen, China) according to the manufacture's protocol. Aliquots of total RNA were subjected to the RT reaction using PrimeScript RT reagent Kit (Takara, Japan). After reverse transcription, qRT-PCR were performed in triplicate in a 96-well plate containing 1 μL of synthesized cDNA, QuantiNova™ SYBR Green PCR Kit (Qiagen, Germany) using StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) according to the manufacturer's instructions. The following primers were used for qRT-PCR: *NFATC1*, Forward: 5'-CCCACTCCTAGTTGCCACAT-3', Reverse: 5'- CCTGCTTTCCTTCTTGTTGG-3'). Fold changes in mRNA expression were calculated using $2^{-\Delta\Delta Ct}$ method and normalized based on GADPH with ABI Step One Software version 2.1. All experiment was repeated for at least three times.

Enzyme-linked immunosorbent assay (ELISA)

ELISA kits (Multi Sciences, Hangzhou, China) were used to determine the supernatant levels of IL-2 in each group at 24 h. The optical density (OD) value was measured at 450 nm on the microplate reader (Bio-Rad, USA). All procedures were followed strictly according to the manufacture's protocols. All experiment was repeated for at least three times.

Cell proliferation assay

The cells were seeded into 96-well plates, at a density of 1×10^4 cells per well, for the cell proliferation assay. Cell proliferation was detected using CCK-8 (Dojindo Molecular Technologies Inc., USA) following to the manufacture's protocols. The 10 μL CCK-8 was added to each well at the 24, 48, 72 and 96 h after stimulation, and then cultured for 4 h. The OD values were read at 450 nm on the microplate reader (Bio-Rad, USA). All experiment was repeated for at least three times.

Western blotting

The protein of cells was extracted using RIPA buffer (Beyotime, China) with PMSF, and quantified by the BCA Protein Assay Kit according to the manufacturer's instructions (Beyotime, China). Protein samples were separated by 10% SDS-PAGE gels, and then transferred onto polyvinylidene fluoride (PVDF) membranes. Following blocking with 5% skimmed milk for 2 h at 24 ℃, the PVDF membranes were incubated with rabbit antibody specific to human NFAT1 (#5861, Cell Signaling Technology, MA, USA, 1:1,000), or GAPDH (#5174, Cell Signaling Technology, MA, USA, 1:1,000), or FLAG (#14793, Cell Signaling Technology, MA, USA, 1:1,000) for overnight at 4 ℃. The membranes were then incubated with anti-rabbit secondary antibody for 2 h at 24 ℃ (Thermo Fisher Scientific Inc., USA, 1:5,000). Chemiluminescence system (Bio-Rad, USA) and Image Lab Software (Bio-Rad Laboratories Inc., USA) were used to analyze the protein expression. All experiment was repeated for at least three times.

Statistical analysis

HWE and MAF analysis were performed using gene frequencies obtained by a single gene counting. We explored the linkage disequilibrium (LD) blocks and tagger SNPs using Haploview version 4.2 software. A genotype association analysis of tagger SNPs was performed using the dominant model (minor allele homozygotes plus heterozygotes *vs.* major allele homozygotes), recessive model (minor allele homozygotes *vs.* heterozygotes plus major homozygotes), additive model (major homozygotes *vs.* heterozygotes *vs.* minor homozygotes), HET model (major homozygotes *vs.* heterozygotes) and HOM model (major homozygotes *vs.* minor homozygotes). Comparisons of the genotypic frequencies between the STA and BPAR groups were assessed using a chi-square test. Genotypic distributions of tagger SNPs between two groups were compared with logistic regression model adjusted for confounding factors identified by general linear model (GLM). Then, significant tagger SNPs were included for further evaluation of the relationship with the pathological changes by using the multivariable logistic regression model. Odds ratios (OR) and 95% confidence intervals (95% CIs) were calculated using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Significant SNPs with BPAR episodes and pathological changes in recipients were selected for the *in vitro* function validation. Results were presented as mean ± standard deviation (SD) from at least three independent experiments using GraphPad Prism 6.0 software (San Diego, CA, USA). Comparisons between mutation and wild-type groups were conducted using an unpaired Student's *t*-test or 2-way ANOVA. A threshold of $P<0.05$ was considered significant. The Bonferroni correction

Table 1 Basic characteristics of patients included in our cohort

Variables	STA group	BPAR group	P value
Case number	131	69	–
Donor age (years; mean ± SD)	42.32±2.05	40.76±3.09	NS
Donor male (%)	61.09	57.97	NS
Recipient age (years; mean ± SD)	38.56±1.40	38.92±1.02	NS
Recipient male (%)	62.60	57.97	NS
Donor type			NS
Living-related	16	7	
DCD	115	62	
Pre-transplant PRA (%; mean ± SD)	0.80±0.46	2.12±0.99	NS
HLA mismatches (>3; %)	85.15	93.48	NS
Immunosuppressive protocol			NS
Pred + MMF + CsA	39	31	
Pred + MMF + TAC	87	19	
Pred + MMF + CsA + SIR	3	3	
Pred + MMF + TAC + SIR	2	16	

BPAR, biopsy-proven acute rejection; STA, stable allograft function; NS, not significant; SD, standard deviation; DCD, donor after cardiac death; PRA, panel reactive antibody; Pred, prednisone; MMF, mycophenolate mofetil; CsA, cyclosporin A; TAC, tacrolimus; SIR, sirolimus.

method was performed to avoid inflation of P value when multiple comparisons happened.

Results

Study population

The baseline characteristics of two study groups are presented in *Table 1*. No significant differences associated with the donor age, donor gender, recipient age, and recipient gender were observed (P>0.05). Among all the enrolled recipients, the majority (88.5%) received donor grafts from a donor after cardiac death (DCD). Although the mean levels of pre-transplant PRA and HLA mismatches, as well as the sirolimus usage proportion in the BPAR group were relatively higher than those in the STA group, no statistical differences were found (P>0.05).

SNP identification

A total of 55 SNPs located in the *NFATC1* gene were identified by TS based on NGS in our study. The genetic information of all detected SNPs was presented in *Table S2*. After HWE and MAF analysis, as well as the removal of SNPs with HWE less than 0.05 and/or minor allele frequencies (MAF <0.05), 20 SNPs were obtained (*Table S3*). Furthermore, 14 tagger SNPs were selected for further association analysis by LD analysis (*Figure 1*).

Association of NFATC1 SNPs with AR episodes

Next, GLM analysis identified two confounding factors, CsA/TAC usage and sirolimus administration (CsA/TAC usage: t=–2.50, P=0.013; sirolimus administration: t=4.79, P<0.001; *Table 2*). Then, a logistic regression analysis with an adjustment for CsA/TAC usage and sirolimus administration was performed for all five analytical models (dominant, recessive, additive, HET and HOM) to explore the relationship between *NFATC1* SNPs and BPAR episodes following kidney transplantation. Overall, four SNPs in the *NFATC1* gene (rs2290154, rs2304738, rs754093 and rs754096) were summarized to be statistically significant between STA and BPAR groups (*Table 3*). We did not observe any significant difference in other tagger SNPs (*Table S4*). In addition, four haplotypes were identified by LD analysis and there was no significant difference with

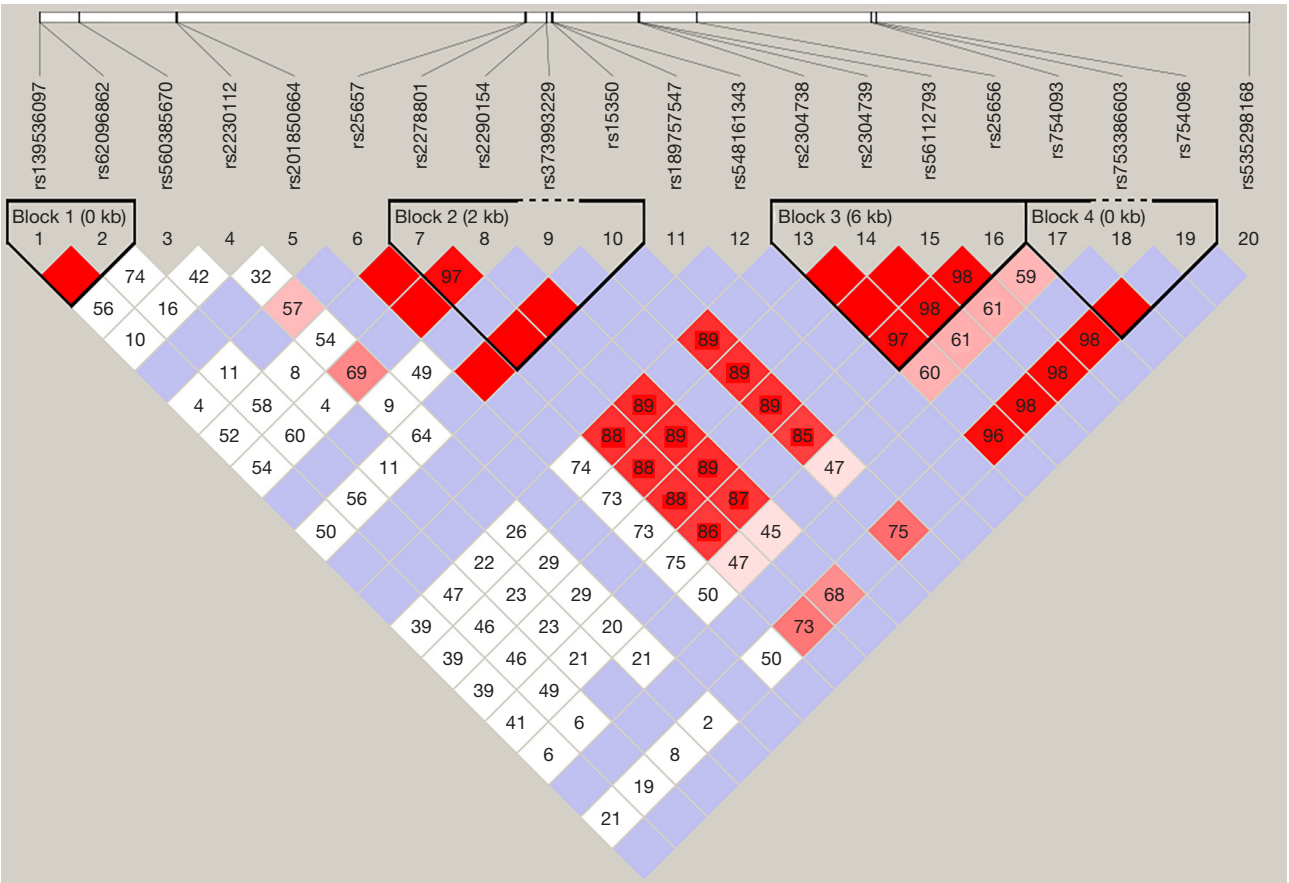

Figure 1 LD results of detected SNPs in the *NFATC1* gene. LD, linkage disequilibrium; SNPs, single nucleotide polymorphisms.

Table 2 Results of clinical confounding factors identification analysis in this cohort using the GLM

Variables	Standardized coefficients	t value	P value
Age	–0.032	–0.48	0.63
Gender	0.036	0.54	0.59
CsA/TAC usage	–0.17	–2.50	0.013
Transplant duration	0.046	0.66	0.51
Sirolimus administration	0.33	4.79	<0.001
DGF episodes	0.13	1.94	0.054

CsA, cyclosporine A; TAC, tacrolimus; DGF, delayed graft function; GLM, general linear model.

BPAR episodes.

Association of NFATC1 SNPs with pathological examination

We examined all 69 recipients suffering from the BPAR episodes and reviewed the each case of pathological slides by two independent pathological professors (H Chen and RY Tan) according to Banff 07 criteria. The results of pathological evaluation were presented in *Table 4*. We explored the relationship between four significant SNPs and pathological changes, and found that only one SNP, rs2290154, was significantly associated with Banff score and t score using multivariable logistic regression (Banff score: t=–3.12, P=0.003; t score: t=2.98, P=0.004; *Table 5*). However, no difference of other three SNPs (rs2304738, rs754093, and rs754096) was observed (*Table S5*).

NFATC1 rs2290154 mutant promotes T cells proliferation and activation

To identify the biological function of rs2290154 mutation on the *NFATC1* gene, we transfected the wild-type and mutated plasmids of rs2290154 into T lymphocytes, respectively. We tested the cell proliferation and found

Table 3 Results of association analysis of four significant taggers SNPs located on *NFATC1* gene with post-transplant BPAR episodes using multivariable logistic regression analysis adjusted by two confounding factors (CsA/TAC usage and sirolimus administration)

SNPs	Model	OR	95% CIs	P value*
rs2290154	Additive	2.2	1.36–3.55	0.001
	Dominant	2.35	1.17–4.74	0.02
	Recessive	3.33	1.47–7.52	0.003
	HOM	1.87	0.90–3.90	0.09
	HET	5.03	1.93–13.13	0.0009
rs2304738	Additive	2.13	1.31–3.45	0.002
	Dominant	2.27	1.18–4.36	0.01
	Recessive	3.35	1.32–8.48	0.01
	HOM	1.89	0.96–3.75	0.06
	HET	4.91	1.76–13.69	0.002
rs754093	Additive	2.30	1.45–3.66	0.0004
	Dominant	2.54	1.29–4.97	0.006
	Recessive	3.64	1.60–8.27	0.002
	HOM	5.56	2.16–14.27	0.0003
	HET	1.98	0.98–4.03	0.05
rs754096	Additive	2.58	1.59–4.22	0.0001
	Dominant	3.44	1.48–7.98	0.004
	Recessive	3.28	1.61–6.68	0.001
	HOM	6.72	2.48–18.14	0.0001
	HET	2.62	1.09–6.26	0.03

*, P value less than adjusted P value (0.05/14=0.0036) was considered as statistical significant. BPAR, biopsy-proven acute rejection; SNPs, single nucleotide polymorphisms; CsA, cyclosporine A; TAC, tacrolimus; OR, odds ratio; CIs, confidential intervals.

Table 4 Pathological evaluation results of 69 recipients in BPAR group of the cohort

Variables*	Results
Case number	69
Banff score#	2.13±1.12
i score	2.43±0.67
g score	0
t score	1.84±0.68
v score	0.22±0.51
ptc score	1.06±0.66

*, data was presented as mean ± SD; #, Banff score was obtained according to the Banff 2007 classification. The i, g, t, v, and ptc scores represent the interstitial inflammation, glomerulitis, tubulitis, intimal arteritis, and peritubular capillaritis, respectively. BPAR, biopsy-proven acute rejection; SD, standard deviation.

Table 5 Results of association analysis of rs2290154 on *NFATC1* gene with the pathological scores obtained from 69 recipients in BPAR group by logistic regression analysis

Variables	Standardized coefficients	t	P value*
i score#	0.10	0.77	0.44
t score	–0.83	–3.12	0.003
v score	–0.54	–2.05	0.045
ptc score	0.20	1.69	0.097
Banff score	1.14	2.98	0.004

*, P value less than adjusted P value (0.05/4=0.0125) was considered as statistical significant; #, Banff score was obtained according to the Banff 2007 classification. The i, g, t, v, and ptc scores represent the interstitial inflammation, glomerulitis, tubulitis, intimal arteritis, and peritubular capillaritis, respectively. BPAR, biopsy-proven acute rejection.

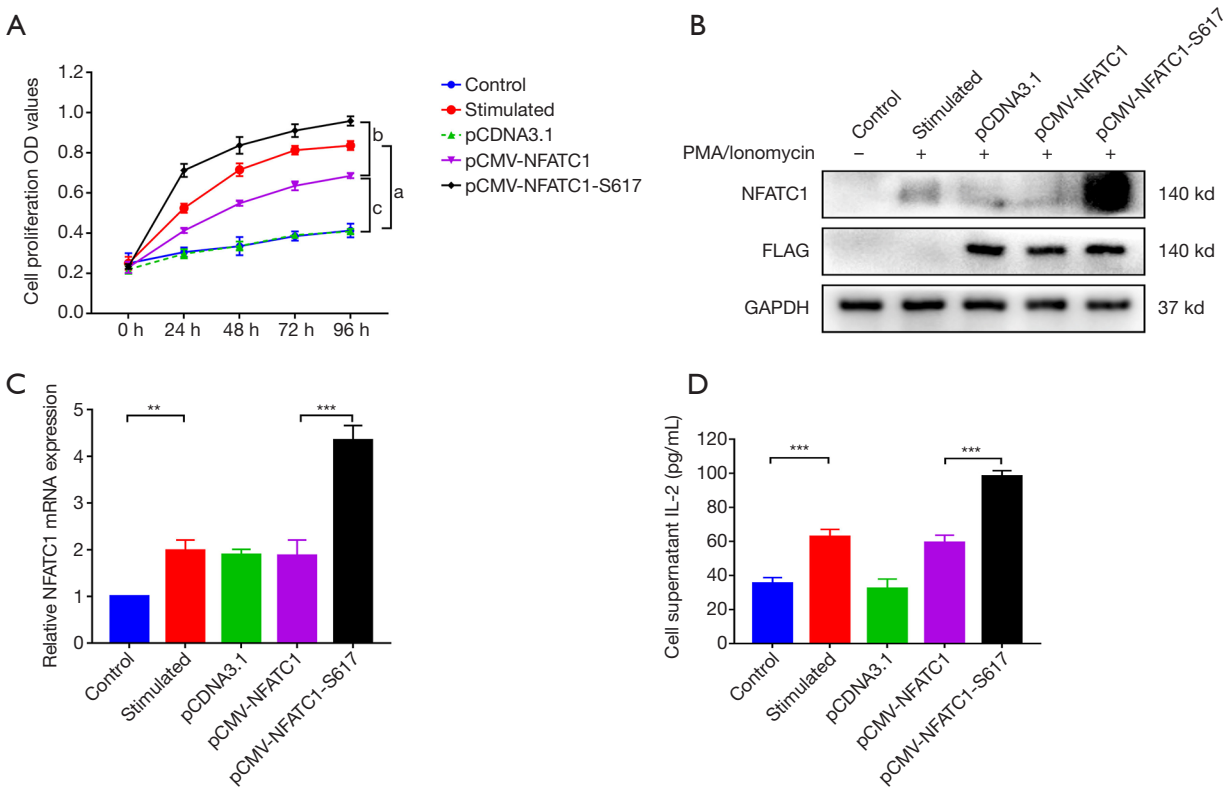

Figure 2 *NFATC1* rs2290154 mutant promotes the T cells proliferation and activation *in vitro*. (A) We used *NFATC1* rs2290154 wild-type and mutation plasmid to transfect the T cells *in vitro* and CCK-8 assay showed that *NFATC1* rs2290154 mutant significantly promoted the T cells proliferation when compared with the wild-type group and stimulation group ([a], P<0.001, stimulated group *vs.* control group; [b], P<0.001, pCMV-NFATC1-S617 group *vs.* pCMV-NFATC1 group; [c], P<0.05, pCMV-NFATC1 group *vs.* pCDNA3.1 group); (B) total proteins from each group were extracted and western blotting results demonstrated the significant increase of NFATC1 protein stimulated by *NFATC1* rs2290154 mutant; (C) total DNA from each group was extracted and *NFATC1* mRNA was tested by RT-PCR. Results indicated the significant increase of *NFATC1* mRNA stimulated by *NFATC1* rs2290154 mutant (data are means ± SD; **, P<0.01; ***, P<0.001); (D) supernatant from cell culture in each group was collected and ELISA was used to examine the concentration of IL-2. We found the statistically increased secretion of IL-2 cytokines after the intervention of *NFATC1* rs2290154 mutation plasmids (data are means ± SD; ***, P<0.001). RT-PCR, real-time PCR; SD, standard deviation; ELISA, enzyme-linked immunosorbent assay; IL-2, interleukin-2.

that *NFATC1* rs2290154 mutant significantly promoted the proliferation of T lymphocytes, especially at 48 and 72 h after stimulation (*Figure 2A*). Then, we examined the translation of *NFATC1* mRNA and expression of NFATC1 protein, the results showed that *NFATC1* mRNA and NFATC1 protein was significantly increased by the *NFATC1* rs2290154 mutant in T lymphocytes (*Figure 2B,C*), along with the remarkable increase of IL-2 in supernatant (*Figure 2D*).

Discussion

Renal transplant recipients are generally exposed to combined immunosuppressive therapy with a limited therapy window, which is associated with a higher rate of BPAR episodes. The *NFATC1* gene was found to influence the susceptibility to AR by altering the activity and expression of NFATC1 protein, which plays an important role in gene transcription for mounting an immune response (25). Here, we conducted an association study of Chinese renal transplant recipients to comprehensively explore the effects of the *NFATC1* gene on the pathogenesis of AR episodes. Sequencing study has identified one SNP, rs2290154, was significantly associated with the BPAR episodes and pathological changes, especially in the Banff score and renal tubulitis. Furthermore, our *in vitro* study has

identified what we believe is a novel molecular mechanism by which *NFATC1* rs2290154 mutant promoted the T lymphocytes activation and proliferation through increasing the translation of *NFATC1* mRNA and expression of NFATC1 protein, and the secretion of IL-2, which is responsible for the increased risk of AR episodes following renal transplant.

It is well established that *NFAT* transcription factors play critical roles in CD4 T cell activation and differentiation and calcineurin/NFAT network has an important function in the generation and function of activated T lymphocytes, including CD4+ and CD8+ T cells (26,27). In one study, a defective nuclear translocation of *NFATC1* has been described for *NFATC1* in CD8+ T cells, not in CD4+ T cells (27). Consistently, ablation of *NFATC1* results in an impaired formation of F-actin rings around the immunological synapse in cytotoxicity T lymphocyte (CTLs) upon TCR stimulation, indicating that *NFATC1* controls the effector function of CD8+ T cells at multiple levels (26). To date, no association studies have been performed to explore the relationship between *NFATC1* gene polymorphisms and the AR episodes that occur following kidney transplantation. Moreover, the mechanisms of such interactions remain unclear. In a clinical study performed in cases of human allograft rejection, an increase in calpain was observed in infiltrating T cells, providing novel prospects for pharmacological manipulation of the calpain/calpastatin balance during solid organ transplantation (28). Since calpain is a calcium-activated protease which is involved in the activation of NF-κB and NFAT, the outcomes in this study indicate that the *NFAT* gene may indirectly participate in the development of rejection. In our study, we sequenced the loci mutations on *NFACT1* gene and identified one SNP mutant may have a positive impact on the NFATC1 protein expression, as well as the cell proliferation, explaining the results observed in our cohort. We provided the genetic evidence of *NFATC1* in the pathogenesis of AR episodes following renal transplant, which could be used as a candidate gene for the regulation of massive T lymphocyte activation and proliferation.

Recently, published GWAS studies summarized that nearly 80% of trait-associated SNPs are located in non-coding regions, such as intron and untranslated region (UTR) (29). Results from numerous studies have implicated crucial regulatory functions to noncoding intronic loci on human genes (30,31). From our sequencing data and *in vitro* results, we have concluded that one intron SNP located in *NFATC1*, rs2290154, may contribute to the significant proliferation and activation of T lymphocytes in AR episodes by the mutation from T allele to C allele. Since no previous studies have associated this mutation with the function of T cells, we were unable to explain the detailed regulatory mechanism of rs2290154 mutant in AR episodes. Furthermore, we have simultaneously verified that two SNPs, rs25657 and rs2278801, in linkage with rs2290154 were not in the chromatin regions annotated as promoters and enhancers. Taken together, our results suggest that the *NFATC1* gene may be a plausible candidate gene for the pathogenesis of post-transplantation AR episodes by encoding a transcription factor (rs2290154 mutant) which regulates gene transcription during an immune response (32).

Some limitations associated with our study should also be acknowledged: (I) this study contained a limited number of subjects from a single transplant center, which inevitably led to the limited power to detect a significant association. Thus, the results needs to be validated by replication in different patient cohorts; (II) there was a lack of information pertaining to the exposure to environmental factors in this study (e.g., diet and lifestyle); (III) our *in vitro* study reveals the positive regulation of *NFATC1* rs2290154 mutant on T cells proliferation, which partly explained the results observed in the occurrence of BPAR episodes of this cohort; and (IV) with regard to the complexity of AR episodes following kidney transplantation, various genetic and non-genetic factors have been identified to partially contribute to the BPAR; while in our study, mainly the genetic factors were explored with the lack of non-genetic factors investigation, which should be interpreted with great caution. However, extensive explore to the influence of this mutant on the T cell activation, differentiation and metabolism are still warrant.

In summary, our study firstly reported the crucial function of *NFATC1* gene in the occurrence of AR episodes. Using the TS based on the NGS technology, we have identified one significant SNP, rs2290154, is significantly associated with the remarkable renal allograft pathological changes. Our *in vitro* study further observed that the *NFATC1* rs2290154 mutant could significantly promoted the T cells proliferation, increased the translation of *NFATC1* mRNA and the expression of NFATC1 protein, as well as the secretion of IL-2. Given the known trigger function of *NFATC1* in T lymphocytes, these findings suggest a possible link between genetic variation in *NFATC1* and the onset of AR following renal transplant. Future studies will be required to validate the importance of *NFATC1* rs2290154 mutant in the progression of AR episodes.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Local ethics committee of the First Affiliated Hospital with Nanjing Medical University approved the protocols followed in this study (2016-SR-029). Written informed consents were obtained from all transplant recipients. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Supplementary

Table S1 Detailed information of *NFATC1* plasmids construction

Vector information
General: 10418bp
ORF frame 1: 1558-2445
EGFP: 4350-5069
Ampicillin: 9425-10285
HIV-1_5_LTR, trunkHIV-1_3_LTR: 835-1015
HIV-1_5_LTR, trunkHIV-1_3_LTR: 6223-6403
CAG_enhancer: 318-605
Psi: 1067-1204
RRE: 1680-1913
cPPT: 2444-2459
H1-F: 2479-2502
3FALG: 3880-3957
WPRE: 5112-5699
pGC-E1-SEQR: 5160-5180
cPPT: 5886-5901
U3PPT: 5886-5907
CMV_immearly_promoter: 239-810
Ubiquitin Promoter: 2617-3833
SV40 promoter: 3964-4341
AmpR_promoter: 10327-10355
pBR322_origin: 8651-9270
Primer locations and sequences
Ubi-F (3756-3778): GGGTCAATATGTAATTTTCAGTG
FLAG-R-1 (3963-3943): GCTAGCTCATTTGTCGTCATC
Carrier Atlas

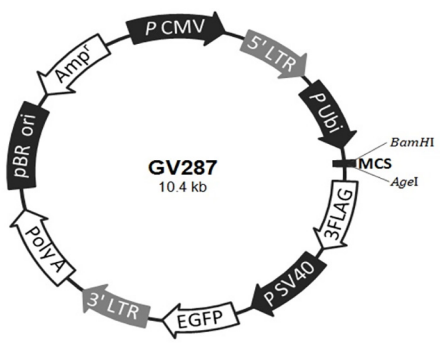

Table S2 Basic information of SNPs located on *NFATC1* gene detected by TS based on NGS

Chromosome	Location	Reference allele	Alternative allele	Function	SNP nomination
chr18	77156171	G	C	UTR5	rs74183647
chr18	77156174	G	A	UTR5	rs139536097
chr18	77156197	C	T	UTR5	rs62096862
chr18	77156449	G	C	Intronic	rs58346397
chr18	77156458	A	G	Intronic	rs8097032
chr18	77160567	C	G	Intronic	rs560385670
chr18	77160601	C	A	Intronic	rs62096871
chr18	77170558	G	A	Exonic	rs781552559
chr18	77170776	C	T	Exonic	rs768935319
chr18	77170857	G	A	Exonic	rs199503337
chr18	77171061	T	G	Exonic	rs2230112
chr18	77171135	C	T	Exonic	rs200533002
chr18	77171164	G	A	Exonic	rs76525142
chr18	77171171	C	T	Exonic	rs201850664
chr18	77171292	C	T	Exonic	rs200853741
chr18	77171313	G	A	Exonic	rs777408035
chr18	77171481	G	C	Exonic	rs200146253
chr18	77171523	G	A	Intronic	rs540117854
chr18	77193717	C	T	Exonic	rs184916992
chr18	77208693	G	A	Intronic	–
chr18	77208735	G	A	Intronic	rs778301024
chr18	77208922	C	T	Exonic	rs25657
chr18	77209015	A	G	Intronic	rs2278801
chr18	77210902	C	T	Intronic	rs680802
chr18	77211175	T	C	Intronic	rs2290154
chr18	77211177	C	T	Intronic	rs373993229
chr18	77211195	G	T	Intronic	rs160191
chr18	77211764	T	C	Exonic	rs15350
chr18	77211795	A	G	Exonic	rs747539233
chr18	77211856	G	A	Intronic	–
chr18	77211872	G	A	Intronic	rs189757547
chr18	77211927	A	G	Intronic	rs548161343
chr18	77211959	G	T	Intronic	–
chr18	77221207	C	A	Intronic	rs2304738
chr18	77221264	C	T	Intronic	rs2304739
chr18	77221278	G	A	Intronic	rs56112793
chr18	77227285	T	C	Intronic	rs2304741
chr18	77227476	A	G	Exonic	rs25656
chr18	77227610	G	A	Exonic	rs55740492
chr18	77246305	C	T	Exonic	rs774688540
chr18	77246406	T	G	Exonic	rs754093
chr18	77246428	T	C	Exonic	rs753386603
chr18	77246444	C	T	Exonic	rs7233684
chr18	77246527	C	T	Exonic	rs200207441
chr18	77246538	G	A	Exonic	rs56191735
chr18	77246548	C	T	Exonic	rs756675542
chr18	77246589	G	A	Exonic	rs201484637
chr18	77246936	C	T	Exonic	rs150249025
chr18	77246977	C	T	Intronic	rs374978531
chr18	77246978	G	A	Intronic	rs754096
chr18	77247051	C	T	Intronic	–
chr18	77287385	T	C	Intronic	rs451691
chr18	77287440	C	G	Intronic	rs535298168
chr18	77287561	C	T	Exonic	rs149271669
chr18	77287776	A	G	UTR3	rs1052025

SNPs, single nucleotide polymorphisms; TS, target sequencing; NGS, next-generation sequencing; UTR, untranslated region.

Table S3 Results of MAF and HWE analysis of all 55 SNPs detected by the TS on *NFATC1* gene based on the genetic frequencies

SNP	MAF	HWE	REF:ALT
rs754096	0.487	0.2683	G:A
rs2290154	0.417	0.2167	T:C
rs2278801	0.407	0.1776	A:G
rs15350	0.4	0.1114	T:C
rs754093	0.395	0.8641	T:G
rs25656	0.372	0.3425	A:G
rs2304738	0.367	0.1794	C:A
rs2304739	0.365	0.2174	C:T
rs56112793	0.365	0.2174	G:A
rs62096871	0.3	2.47E–53	C:A
rs74183647	0.268	7.36E–05	G:C
rs2230112	0.133	0.5831	G:T
rs58346397	0.125	5.31E–33	G:C
rs62096862	0.105	0.3049	C:T
rs139536097	0.095	0.1654	G:A
rs25657	0.075	1	C:T
rs8097032	0.035	1.87E–13	A:G
rs560385670	0.018	1	C:G
rs451691	0.013	2.00E–04	T:C
rs373993229	0.007	1	C:T
rs535298168	0.007	1	C:G
rs201850664	0.005	1	C:T
rs189757547	0.005	1	G:A
rs548161343	0.005	1	A:G
rs753386603	0.005	1	T:C
rs781552559	0.003	1	G:A
rs768935319	0.003	1	C:T
rs199503337	0.003	1	G:A
rs200533002	0.003	1	C:T
rs76525142	0.003	1	G:A
rs200853741	0.003	1	C:T
rs777408035	0.003	1	G:A
rs200146253	0.003	1	G:C
rs540117854	0.003	1	G:A
rs184916992	0.003	1	C:T
Chr18:77208693	0.003	1	G:A
rs778301024	0.003	1	G:A
rs747539233	0.003	1	A:G
Chr18:77211856	0.003	1	G:A
Chr18:77211959	0.003	1	G:T
rs2304741	0.003	1	T:C
rs55740492	0.003	1	G:A
rs774688540	0.003	1	C:T
rs7233684	0.003	1	C:T
rs200207441	0.003	1	C:T
rs56191735	0.003	1	G:A
rs756675542	0.003	1	C:T
rs201484637	0.003	1	G:A
rs150249025	0.003	1	C:T
rs374978531	0.003	1	C:T
Chr18:77247051	0.003	1	C:T
rs149271669	0.003	1	C:T
rs1052025	0.003	1	A:G
rs680802	0	1	T:T
rs160191	0	1	T:T

MAF, major allele frequency; HWE, Hardy-Weinberg equilibrium; TS, target sequencing; SNPs, single nucleotide polymorphisms.

Table S4 Results of association analysis of 10 tagger SNPs on *NFATC1* gene with post-transplant BPAR episodes using multivariable regression analysis adjusted by CsA/TAC usage and sirolimus administration

SNPs	Model	OR	95% CIs	P value
rs139536097	Additive	0.97	0.49–1.91	0.94
	Dominant	1.03	0.47–2.25	0.95
	Recessive	0.61	0.06–6.15	0.67
	HOM	0.62	0.06–6.25	0.68
	HET	1.09	0.48–2.49	0.83
rs560385670	Additive	0.28	0.03–2.41	0.24
	Dominant	0.28	0.03–2.41	0.24
rs2230112	Additive	0.81	0.42–1.57	0.54
	Dominant	0.76	0.38–1.52	0.43
	Recessive	2.35	0.14–39.13	0.55
	HOM	2.18	0.13–36.45	0.59
	HET	0.71	0.35–1.47	0.36
rs201850664	Additive	1.54	0.09–25.96	0.77
	Dominant	1.54	0.09–25.96	0.77
rs25657	Additive	0.69	0.29–1.62	0.39
	Dominant	0.71	0.29–1.70	0.44
	HET	0.74	0.31–1.79	0.50
rs373993229	Additive	0.88	0.07–10.29	0.92
	Dominant	0.88	0.07–10.29	0.92
rs189757547	Additive	1.06	0.063–18.06	0.97
	Dominant	1.06	0.063–18.06	0.97
rs535298168	Additive	1.47E–09	–	0.99
	Dominant	1.47E–09	–	0.99
rs548161343	Additive	1.52E–09	–	0.99
	Dominant	1.52E–09	–	0.99
rs753386603	Additive	7.43E–10	–	0.99
	Dominant	7.43E–10	–	0.99

*, P value less than adjusted P value (0.05/14=0.0036) was considered as statistical significant. BPAR, biopsy-proven acute rejection; SNPs, single nucleotide polymorphisms; OR, odds ratio; CIs, confidential intervals; CsA, cyclosporine A; TAC, tacrolimus.

Table S5 Results of association analysis of three significant tagger SNPs with the pathological scores of 69 recipients in BPAR group using multivariable logistic regression analysis

Variables	Standardized coefficients	t	P value*
rs2304738			
i score#	–0.12	–0.93	0.36
t score	0.59	2.23	0.029
v score	0.41	1.55	0.13
ptc score	–0.29	–2.45	0.017
Banff score	–0.86	–2.26	0.027
rs754096			
i score	0.003	0.02	0.98
t score	0.28	0.99	0.32
v score	0.13	0.46	0.65
ptc score	–0.28	–2.25	0.028
Banff score	–0.38	–0.94	0.35
rs754093			
i score	0.22	1.57	0.12
t score	0.26	0.94	0.35
v score	0.23	0.82	0.41
ptc score	–0.15	–1.19	0.24
Banff score	–0.58	–1.45	0.15

*, P value less than adjusted P value (0.05/4=0.0125) was considered as statistical significant; #, Banff score was obtained according to the Banff 2007 classification. The i, g, t, v, and ptc scores represent the interstitial inflammation, glomerulitis, tubulitis, intimal arteritis, and peritubular capillaritis, respectively. SNPs, single nucleotide polymorphisms; BPAR, biopsy-proven acute rejection.